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Note

Characterization of buffalo lactotransferrin by polyacrylamide gel electrophoresis in 6 *M* urea

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Lactotransferrin is a transferrin-like iron-binding protein present in the milk and other secretions of mammals. The iron-binding proteins, such as lactotransferrin, ovotransferrin and transferrin, are characteristically composed of a single polypeptide chain. The molecular mass falls between 70 000 and 80 000. These proteins bind two iron atoms per molecule at two iron-binding sites. For each iron(III) bound, one molecule of bicarbonate is incorporated into the complex [1,2]. The most apparent difference between these metal-binding proteins is their relative affinity for iron and the conditions necessary for in vitro removal of iron. The lactotransferrins (bovine and human milks) apparently have a higher affinity for iron than ovotransferrin and transferrin [3]. Furthermore, removal of iron from lactotransferrin requires exposure to pH 2, whereas metal-free forms of transferrin and ovotransferrin can be prepared at pH 4.

The blood serum transferrin molecule has been separated into four molecular forms by electrophoresis. The separation of these forms correlates directly with the amount of iron bound by the transferrin: iron-free transferrin, iron bound only to the C-terminal binding site (A-site) or only to the N-terminal binding site (B-site) and iron bound to both binding sites. The molecular basis for the electrophoretic separation of transferrin into four molecular forms is complex and incompletely defined, but may be due to structural differences between the domains resulting in unequal binding of metals to the two sites.

No reports are available regarding electrophoretic characterization and resolution of the sites of lactotransferrin as there are for other transferrin-like molecules. The present study was therefore carried out to discover whether the two lactotransferrin sites can also be characterized by electrophoresis. The method

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of Makey and Seal [4] and other methods [5–7] at various pH values were employed. A new modified method is reported for the resolution and characterization of the sites of lactotransferrin.

EXPERIMENTAL

All glassware was treated with Teepol (BDH, India) and AR concentrated nitric acid (Merck, India), and then thoroughly rinsed with "Milli-Q" water (Millipore, U.S.A.) before use to minimize metal ion contamination.

Buffalo lactotransferrin was isolated from buffalo colostrum of the Murrah herd maintained at the National Dairy Research Institute (Karnal, India). Skimmed colostrum diluted three times with water was adjusted to pH 4.6 using 1 *M* hydrochloric acid (Merck), and lactotransferrin was prepared and purified according to the method of Law and Reiter [8] using CM-Sephadex C-50 and Sephadex G-200 (Pharmacia, Sweden). A few preparations were also prepared by affinity chromatography on Heparin-Ultrogel A 4R (LKB, Sweden) [9]. The purity of the lactotransferrin was confirmed by immunoelectrophoresis (LKB Application Note 249) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [10]. The equipment used was an LKB 2117 Multiphor-II and an LKB 2001 vertical electrophoresis unit. Antiserum for immunoelectrophoresis was raised in rabbits using Freund's complete adjuvant (Sigma, U.S.A.) in foot pads followed by weekly intravenous injections of the protein for four weeks in the ear vein. Apolactotransferrin, the metal-free form of lactotransferrin, was prepared according to Masson et al. [11]. The iron-saturated form of lactotransferrin was prepared by using citrate–bicarbonate (0.1 *M*) and ferric chloride hexahydrate according to Mazurier and Spik [12], and was found to be 98% saturated with iron.

Individual, immunologically pure preparations of apolactotransferrin, saturated lactotransferrin and native lactotransferrin were used to test their nature.

Electrophoresis

Vertical gel electrophoresis was carried out using an LKB 2001 vertical electrophoresis unit equipped with 2301-Macrodrive-1 and 2219-Multitemp-II (LKB).

Electrophoresis was carried out in 6 *M* urea as follows. The electrode buffer (0.043 *M* sodium bicarbonate and 0.052 *M* sodium carbonate) was prepared in Milli-Q water; the final pH was adjusted to 10.0, and the ionic concentration was 0.2. To prepare the sample buffer, 0.1 ml of 1% Bromophenol blue (BDH, U.K.) in water was added to 18 g of urea, and the volume was made up to 100 ml with electrode buffer. The gel solution contained 5.79 g of polyacrylamide (LKB), 0.30 g of N,N′-methylenebisacrylamide (LKB) and 36 g of urea. The final volume was made up to 100 ml with electrode buffer. Amido black 10 B (1%, Merck) in methanol–water–acetic acid (4:4:1) was used to stain the gels for 10 min. The gels were destained in methanol–water–acetic acid (2:4:1) by repeated changes till free from dye.

Gel of 1.5 mm thickness was prepared and pre-electrophoresed for 1 h. The

samples were then applied at a constant current of 1.5 mA/cm of gel slab; 10–20 μl of sample were applied, depending on the number of bands expected. After sample application a final current of 2.5 mA/cm of gel slab was applied. Electrophoresis was carried out at 10°C for 14–16 h.

RESULTS AND DISCUSSION

Lactotransferrin resolved into three distinct bands, depending on the iron content. Apolactotransferrin moved as a single band and showed the slowest mobility (Fig. 1).

Native lactotransferrin resolved into three bands. These bands corresponded to the apo, intermediate and saturated forms of lactotransferrin. The saturated lactotransferrin migrated as a single band with the fastest electrophoretic mobility.

Lactotransferrin did not move at all in the Makey and Seal [4] electrophoresis system (pH 8.4) developed for transferrin. Lactotransferrin samples remained at the top of the gel slot. This suggests that lactotransferrin and transferrin behave differently under these conditions. Thereafter, a pH range of 8.4–9.5 was employed for electrophoresis using different buffers (Tris–boric acid, Tris–boric acid–EDTA and Tris–HCl), but no clear resolution could be obtained. Finally bicarbonate–carbonate buffer (pH 10.0, $I=0.2$) was employed, and lactotransferrin resolved into three components. On reducing the ionic strength the clarity of the resolution diminished.

In the case of native lactotransferrin, the three bands correspond to the three

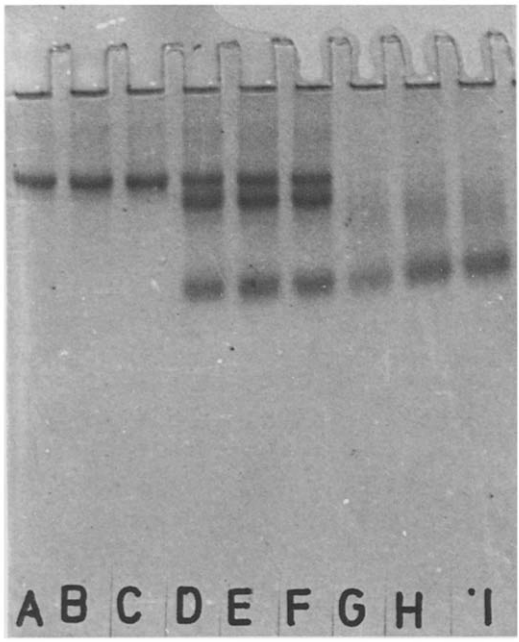

Fig. 1. Electrophoresis of lactotransferrin in 6 *M* urea at pH 10.0, $I=0.2$: (A, B, C) apolactotransferrin; (D, E, F) native lactotransferrin; (G, H, I) saturated lactotransferrin.

molecular form of lactotransferrin with different iron content. The slowest one coincided with apolactotransferrin and the fastest matched saturated lactotransferrin. The one intermediate form represents the molecular form of protein in which, perhaps, the N and C terminal sites are both partially occupied by iron. Individual characterization of the two sites (N and C terminal) was not possible by this procedure, as it is in the case of transferrin in which the two iron-binding sites show different mobility [4]. The reason for this distinct behaviour may be that the iron atom in both the binding sites of lactotransferrin is either filled or removed simultaneously without showing the differential resolution in the two sites as in case of transferrin. The different electrophoretic behaviour of transferrin and lactotransferrin indicates that the two molecules have distinct iron-binding properties and that they require different conditions for resolution into their molecular forms.

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